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# Resveratrol-loaded nanocarriers: Formulation, optimization, characterization and *in vitro* toxicity on cochlear cells

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ABSTRACT

The present work aimed to investigate the suitability of polymeric nanoparticles (NPs) loaded with resveratrol (RES) for drug delivery to cochlear cells. RES-loaded NPs were prepared by a solvent-diffusion method without surfactant. The Box–Behnken design was used to study the effect of the formulation variables on the particle mean diameter (PMD), polydispersity index (PDI), zeta-potential ($\zeta$), percent drug encapsulation efficiency (EE%), and ratio between NP size before and after freeze-drying ($S_f/S_i$). The physicochemical stability of the RES-loaded NPs during freeze-drying was investigated using four well-known cryoprotectants (*i.e.*, lactose, mannitol, sucrose, and trehalose) at different concentrations. The RES-loaded NPs were also characterized by powder X-ray diffraction (PXRD) and *in vitro* drug release studies. Finally, the *in vitro* toxicity of the synthesized NPs was evaluated on two cochlear cell lines: HEI-OC1 and SVK-1 cells. The optimal formulation (desirability: 0.86) had $135.5 \pm 37.3$ nm as PMD, $0.126 \pm 0.080$ as PDI, $-26.84 \pm 3.31$ mV as $\zeta$, $99.83 \pm 17.59$% as EE%, and $3.30 \pm 0.92$ as $S_f/S_i$ ratio. The PMD and PDI of the RES-loaded NPs were maintained within the model space only when trehalose was used at concentrations higher than 15% (w/v). Results from the *in vitro* cytotoxicity studies showed that blank NPs did not alter the viability of both cells lines, except for concentrations higher than 600 μg/mL. However, the cell viability was significantly decreased at high concentrations of native RES (>50 μM, $p < 0.05$) in both cell lines. Overall, the results suggested that the RES-loaded polymeric NPs could be a suitable template for cochlea antioxidant delivery and otoproctection.



## 1. Introduction

Cisplatin (cis-diaminedichloroplatinum (II)) is one of the most frequently used chemotherapeutic agents for the treatment of several types of tumors, including ovarian, testicular, breast, hematologic, lung, cervical, and head and neck cancers.

However, its clinical effectiveness is often correlated with severe side effects such as ototoxicity [1]. Cisplatin-induced ototoxicity generally manifests as ear pain, tinnitus, sensorial hearing loss, and deafness. In particular, around 60% of treated patients exhibited an elevated hearing threshold, with a higher incidence in children [2].

Cisplatin has been demonstrated to trigger *in vitro* the activation of apoptosis in cochlear cells (*i.e.*, outer hair, strial ganglion, and stria vascularis cells) through the generation of reactive oxygen species (ROS) [3,4]. Even if the efficacy of several antioxidant agents has been proven *in vitro* and *in vivo* after local or systemic administration [1,5], no FDA/EMA-approved medicines are currently available for the treatment of hearing impairment caused by cisplatin.

Recently, resveratrol (RES) has been proposed as a protective agent since its effectiveness has been proven after systemic administration in guinea pigs and rats [6,7]. RES (3,5,4′-trihydroxystilbene) is a non-flavonoid polyphenol antioxidant compound abundant in grapes, peanuts, and other foods. In humans, RES has been associated with several pharmacological effects, including cardioprotection, cancer prevention, anti-inflammatory activity, antioxidant effects, an increase of cellular stress resistance, and longevity [8]. However, several delivery systems, including microparticles, ultra-fine fiber, nanosponges, liposomes, and nanoparticles (NPs) have been proposed as alternative

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solutions to bypass the pharmacokinetic limitations to therapeutic use of RES [9].

In the present study, RES-loaded NPs were made of biodegradable polymers. Poly-(lactic-co-glycolic) acid (PLGA) was selected for its well-known safety and ability to control drug release kinetics. On the other hand, poly(ε-caprolactone)–poly(ethylene glycol) (PCL–PEG) di-block was used to take advantage of its amphiphilic nature and stabilize the NPs surface while improving its drug loading.

Experimental approaches based on design of experiment (DoE) have been widely applied in academic research and pharmaceutical development of new drug products [10,11]. For this purpose, Box–Behnken design (BBD) was selected for the optimization of the RES-loaded NPs. BBD offers some advantages in requiring only three levels of each factor and a smaller number of runs than other experimental designs for response surface methodology [12]. Such an approach allows for deep investigation of the influence of each formulation variable (*i.e.*, RES, PLGA and PCL-PEG amounts) on the selected responses, namely particle mean diameter (PMD), PDI, zeta-potential (ζ), percent drug encapsulation efficiency (EE%), and physicochemical changes after freeze-drying. The optimal formulation proposed by BBD was also characterized by powder X-ray diffraction (PXRD), scanning electron microscopy (SEM), and *in vitro* drug release. Furthermore, the effects of well-known cryoprotectants were investigated to protect the nanomaterials from the freezing stress and to minimize the physical instability of NPs during the freeze-drying process.

So far, no information is available on the pharmacology and toxicology of RES in cochlear cells. Therefore, its toxicity was evaluated *in vitro* in two different cochlear cell lines: organ of Corti cells (HEI-OC1) and a stria vascularis one (SVK-1) [13,14]. The toxicity of blank and RES-loaded NPs was also tested in HEI-OC1 and SVK-1 cells.

## 2. Materials and methods

### 2.1. Materials

The RES was obtained from AK Scientific (AK Scientific, Inc. Union City, CA). Poly(D,L-lactide-co-glycolide) with a L/G ratio of 50:50 and inherent viscosity of 0.39 dL/g was supplied from Birmingham Polymers (Birmingham Polymers Inc., Pelham, AL). The poly(ε-caprolactone)–poly(ethylene glycol) di-block (5–10 kDa) was obtained from Advanced Material Polymers (Advanced Material Polymers Inc., Montréal, Canada). The sucrose, trehalose, and methanol were supplied from Sigma Aldrich (Sigma Aldrich, Saint-Louis, MO). The lactose, mannitol, and acetone were obtained from Fisher Scientific (Fisher Scientific Inc., Pittsburgh, PA). All other chemicals were of analytical grade and used without further purification.

### 2.2. Preparation of resveratrol-loaded nanoparticles

The RES-loaded NPs were prepared by adapting a solvent-diffusion technique without surfactant [15]. The NPs were prepared by varying the amounts of RES, PCL-PEG, and PLGA (Table 1). Briefly, a known amount of PLGA and PCL-PEG was dissolved in 1 mL of acetone containing a known amount of RES. The resulting solution was added dropwise into 10 mL of purified water (Direct-Q 3 UV system, Millipore SAS, Molsheim, France) under constant mechanical stirring at 1100 rpm (stirrer model RO 15, IKA-WerkeGmbh & Co, Staufen, Germany). The organic phase was evaporated (BUCHI Labor technik AG, Flawil, Switzerland) at 25 °C for 2 h. Finally, the RES-loaded NPs were isolated by centrifugation at 15,000 rpm and 4 °C for 20 min (VWR International Micro 18R, Darmstadt, Germany), washed twice, and re-suspended in purified water. The suspension was frozen at −196 °C in liquid nitrogen and dried at −47 °C and 0.01 mbar for 24 h (Labconco Corporation, Kansas City, MO). The powdered RES-loaded NPs were stored at 4 °C until used. Blank NPs were similarly prepared without the active substance.

### 2.3. Preparation of resveratrol nanocrystals

The RES nanocrystals were produced according to a published method [16] using acetone and water as the solvent and anti-solvent, respectively. The solvent containing RES and the anti-solvent were mixed rapidly to assure fast nucleation, thereby forming small particles. The RES nanocrystals were collected by centrifugation (10,000 rpm for 20 min at 5 °C), washed twice, and then freeze-dried.

### 2.4. Design of experiments

#### 2.4.1. Experimental design

The BBD was used as experimental design for response surface methodology to optimize the composition of the RES-loaded NPs. The formulation variables such as RES ($X_1$), PLGA ($X_2$), and PCL–PEG amounts ($X_3$) were selected as independent variables (factors). The amount of drug and polymers ranged from 3 mg (coded level: −1) to 9 mg (coded level: +1). The particle mean diameter (PMD, $Y_1$), polydispersity index (PDI, $Y_2$), zeta-potential (ζ, $Y_3$), percent drug encapsulation efficiency (EE%; $Y_4$), and ratio between NPs diameters before ($S_i$) and after ($S_f$) freeze-drying ($S_f/S_i$, $Y_5$) were used

**Table 1**
Experimental matrix, observed responses from randomized runs, optimal formulation ($R_{16}$), and checkpoint formulation ($R_{17}$, $R_{18}$) in the BBD.

| Form. | $X_1$ (mg) | $X_2$ (mg) | $X_3$ (mg) | $Y_1$ (d-nm) | $Y_2$ | $Y_3$ (mV) | $Y_4$ (%) | $Y_5$ |
|---|---|---|---|---|---|---|---|---|
| $R_1$ | 6 | 9 | 3 | 203.4 | 0.089 | −33.00 | 104.18 | 3.55 |
| $R_2$ | 3 | 3 | 6 | 128.0 | 0.152 | −29.03 | 31.11 | 4.07 |
| $R_3$ | 3 | 9 | 6 | 152.7 | 0.129 | −35.20 | 47.43 | 4.68 |
| $R_4$ | 3 | 6 | 9 | 138.5 | 0.147 | −30.83 | 43.95 | 5.84 |
| $R_5$ | 9 | 9 | 6 | 329.5 | 0.334 | −26.00 | 103.08 | 3.01 |
| $R_6$ | 6 | 3 | 3 | 152.7 | 0.129 | −28.50 | 98.52 | 4.68 |
| $R_7$ | 6 | 3 | 9 | 119.1 | 0.194 | −21.15 | 138.45 | 2.34 |
| $R_8$ | 9 | 6 | 3 | 212.5 | 0.157 | −31.77 | 107.5 | 4.62 |
| $R_9$ | 6 | 6 | 6 | 164.8 | 0.137 | −28.77 | 77.62 | 3.79 |
| $R_{10}$ | 6 | 9 | 9 | 178.4 | 0.215 | −26.93 | 116.82 | 3.89 |
| $R_{11}$ | 9 | 6 | 9 | 289.4 | 0.520 | −12.83 | 141.35 | 1.69 |
| $R_{12}$ | 6 | 6 | 6 | 164.6 | 0.133 | −27.20 | 95.04 | 3.50 |
| $R_{13}$ | 3 | 6 | 3 | 167.6 | 0.162 | −22.33 | 63.99 | 2.73 |
| $R_{14}$ | 6 | 6 | 6 | 177.7 | 0.202 | −23.93 | 90.15 | 2.67 |
| $R_{15}$ | 9 | 3 | 6 | 161.5 | 0.180 | −22.97 | 100.18 | 2.45 |
| $R_{16}$ | 7.4 | 3 | 5.3 | 136.2 | 0.127 | −26.80 | 100.09 | 3.30 |
| $R_{17}$ | 7.5 | 7.5 | 7.5 | 236.70 | 0.289 | −24.47 | 100.65 | 2.46 |
| $R_{18}$ | 4.5 | 4.5 | 4.5 | 136.10 | 0.137 | −27.20 | 75.32 | 4.31 |